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Early impact of social isolation and breast tumor progression in mice

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ABSTRACT

Evidence from cancer patients and animal models of cancer indicates that exposure to psychosocial stress can promote tumor growth and metastasis, but the pathways underlying stress-induced cancer pathogenesis are not fully understood. Social isolation has been shown to promote tumor progression. We examined the impact of social isolation on breast cancer pathogenesis in adult female severe combined immunodeficiency (SCID) mice using the human breast cancer cell line, MDA-MB-231, a high β-adrenergic receptor (AR) expressing line. When group-adapted mice were transferred into single housing (social isolation) one week prior to MB-231 tumor cell injection into a mammary fat pad (orthotopic), no alterations in tumor growth or metastasis were detected compared to group-housed mice. When social isolation was delayed until tumors were palpable, tumor growth was transiently increased in singly-housed mice. To determine if sympathetic nervous system activation was associated with increased tumor growth, spleen and tumor norepinephrine (NE) was measured after social isolation, in conjunction with tumor-promoting macrophage populations. Three days after transfer to single housing, spleen weight was transiently increased in tumor-bearing and non-tumor-bearing mice in conjunction with reduced splenic NE concentration and elevated CD11b + Gr-1+ macrophages. At day 10 after social isolation, no changes in spleen CD11b+ populations or NE were detected in singly-housed mice. In the tumors, social isolation increased CD11b + Gr-1+, CD11b + Gr-1-, and F4/80+ macrophage populations, with no change in tumor NE. The results indicate that a psychological stressor, social isolation, elicits dynamic but transient effects on macrophage populations that may facilitate tumor growth. The transiency of the changes in peripheral NE suggest that homeostatic mechanisms may mitigate the impact of social isolation over time. Studies are underway to define the neuroendocrine mechanisms underlying the tumor-promoting effects of social isolation, and to determine the contributions of increased tumor macrophages to tumor pathogenesis.

1. Introduction

The emotional stress experienced by cancer patients can be associated with increased tumor progression (Antoni et al., 2006), but the biological pathways involved in stress-induced tumor progression are only beginning to be understood. In animal models of cancer, exposure to stressors potentiates tumor growth and metastasis in a variety of tumors (Hermes et al., 2009; Saul et al., 2005; Shakhar and Ben-Eliyahu, 1998; Sloan et al., 2010; Thaker et al., 2006; Williams et al., 2009) suggesting that therapies targeting stress biochemical pathways may be effective in reducing tumor progression. Here we examine the impact of social isolation of adult mice, an ethologically relevant stressor, on breast tumor growth. Social isolation in rodents elicits anxiety and other fearful behaviors (Hermes et al., 2009; Williams et al., 2009). Furthermore, chronic social isolation as experienced by humans has been linked to cancer (Reynolds and Kaplan, 1990), and is a risk factor for cancer mortality and other diseases (Hawkley and Cacioppo, 2003).

The sympathetic nervous system (SNS) is a major stressor pathway characterized by release of the catecholamines norepinephrine (NE) and epinephrine (EPI) from sympathetic noradrenergic nerves and from the adrenal medulla. Several lines of evidence point to a role for the SNS in modulating tumor progression. Regional ablation of sympathetic nerves depleted NE and reduced tumor growth (Raju et al., 2007). Stress-induced increase in tumor growth and/or metastasis can be prevented by pre-treatment with a β-AR blocker prior to stressor exposure or mimicked using β-AR agonists *in vivo* (Shakhar and Ben-Eliyahu, 1998; Sloan et al., 2010; Thaker et al., 2006). Furthermore, using an ovarian cancer model, Thaker and colleagues showed that β-AR expression by the tumor cells was necessary for stressor-induced tumor growth (Thaker et al., 2006). It is interesting to note the variety of targets in stress- and

Abbreviations: IL-6, Interleukin 6; SNS, sympathetic nervous system; NE, norepinephrine; EPI, epinephrine; β-AR, beta-adrenergic receptors; VEGF, vascular endothelial growth factor.

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or SNS-induced potentiation of tumor progression including cells of the immune system (for example, macrophages and natural killer cells) (Shakhar and Ben-Eliyahu, 1998; Sloan et al., 2010), angiogenesis (Thaker et al., 2006), and direct stimulation or inhibition of tumor proliferation (Slotkin et al., 2000). In addition to understanding the stress-induced neuroendocrine mediators/receptors that modulate tumor pathogenesis, it is also important to identify the target cells in order to predict the outcome of stress exposure and to develop therapies with minimal side effects.

The MDA-MB-231 cell line is a human mammary tumor adenocarcinoma representative of the more aggressive triple negative human breast cancer. MB-231 cells express high levels of β-AR, as detected by standard radioligand binding assay, but other breast cancer cell lines displayed a low level of β-AR expression and minimal responsiveness to NE *in vitro* (Madden et al., 2011). By contrast, NE stimulation of MB-231 cells inhibits VEGF production, and dramatically increases IL-6 production *in vitro*. With the view that MB-231 serves as a model of breast cancers expressing high levels of β-AR, we have begun testing the impact of stressor exposure on MB-231 tumor growth, using social isolation as an established model of a psychological stressor that can promote tumor progression, including spontaneous mammary tumor progression (Hermes et al., 2009; Thaker et al., 2006; Williams et al., 2009).

We report here that social isolation transiently increased tumor growth only when social isolation was initiated when tumors were palpable. These changes were associated with alterations in tumor macrophage populations early after social isolation and not associated with elevated tumor or peripheral NE concentration. The results demonstrate the complexity of the response of breast tumors to stressor exposure that needs to be better characterized before targeting stress hormones in the therapeutic treatment of breast cancer.

2. Materials and methods

2.1. Mice

Female severe combined immunodeficiency (SCID) (NOD.CB17-*Prkdc*^{scid}/J) mice were purchased from The Jackson Laboratory, Bar Harbor, ME between 6 and 8 weeks of age, and were housed 5 per cage with food and water ad lib on a 12:12 light:dark cycle. SCID mice were provided acid water ad lib upon arrival. The mice were housed using microisolator technology to effect a biological barrier at the level of the individual cage. Upon initiation of the experiments, the antibiotic sulfamethoxazole and trimethoprim (Hi-Tech Pharmacol. Co., Amityville, NY) was included in the drinking water throughout the duration of the experiment. The antibiotic treatment was necessary in order to prevent the occasional pneumonia that developed in these immunodeficient mice. All experimental protocols were approved by the University of Rochester University Committee on Animal Resources.

2.2. Cell lines

MB-231 tumor cells (American Tissue Type Collection; Manassas, VA) were maintained in Dulbecco's Modified Essential Medium (DMEM) containing 4.5 g/L glucose, L-glutamine, penicillin/streptomycin and 10% fetal calf serum (FCS) (all from Gibco, Invitrogen Inc., Carlsbad, CA). MB-231 cells were employed experimentally within 3 months of acquiring and/or thawing, and were regularly tested for the absence of mycoplasma contamination.

2.3. Social isolation

SCID mice were allowed to adapt to group housing (5 per cage) for at least two weeks before being housed singly. Both group- and single-housed mice were housed in cages measuring 7.5" × 11" × 5".

2.4. Tumor implantation and measures

MB-231 ($2–4 \times 10^6$ cells) was injected into a single mammary fat pad of NOD/SCID female mice in mice anesthetized with 90 mg/kg ketamine and 9 mg/kg xylazine. Mice were palpated weekly until tumors were detected. The shortest and longest diameters of each tumor were measured with calipers. Tumor volumes was calculated using the equation: $0.5 \times \text{length} \times \text{width}^2$.

2.5. Flow cytometry

Single suspensions from spleen or tumors were prepared by pressing tissue through a metal mesh into ice-cold PBS containing 10% fetal calf serum. Red blood cells were lysed using ammonium lysis buffer. After washing, the cells were counted and resuspended in phosphate buffered saline containing 1% bovine serum albumin and 0.25% sodium azide (flow wash). Macrophages were stained using three-color immunofluorescence. Cells (1.5×10^6) were incubated in 25 μl FcBlock (anti-CD16, diluted 1:50; BD Biosciences, bdbiosciences.com) for 15 min at 4 °C. Rat anti-F4/80 (clone BM8; FITC-conjugated; Abcam Inc.; abcam.com), rat anti-CD11b (clone M1/70; Alexafluor 647-conjugated, BD Biosciences) and rat anti-Gr-1 (anti-Ly-6G and Ly-6C; clone RB6-8C5; PE-conjugated; BD Biosciences) were diluted 1:50 in flow wash. Antibodies (100 μl) were incubated 30 min at 4 °C. Cells incubated in flow wash only served as autofluorescent controls. Cells were washed two times in flow wash, fixed in 0.5 ml PBS containing 1% paraformaldehyde, and stored in the dark at 4 °C for no longer than 2 weeks before analysis. Fluorescence was analyzed in the University of Rochester Flow Cytometry Core on a BD LSR II 18-Color flow cytometer. Forward scatter and side scatter gating was used to eliminate non-lymphoid cells from the analysis. Analysis gates were set based on the autofluorescent controls.

2.6. Cytokine and norepinephrine determination

For cytokines, tumor homogenates at a concentration of 4% w/v were prepared in RIPA buffer containing protease inhibitors (HALT Protease Inhibitor Cocktail, Thermoscientific; Thermofisher.com). To measure NE, tumors were homogenized at a concentration of 1% w/v in 1 N HCl. Protein in homogenates was measured colorimetrically using a Pierce BCA Protein Assay kit (Thermoscientific). NE and tumor cytokines in the homogenates were measured by ELISA according to the manufacturer's instructions. A NE ELISA kit was purchased from Rocky Mountain Diagnostics. Mouse- and human-specific VEGF and IL-6 Quantikine kits (R and D Systems, Minneapolis, MN) are highly species specific with little or no cross-reactivity detected with the corresponding analyte from other species. As reported by the manufacturer and confirmed in our laboratory, the only (minimal) cross-reactivity detected is 0.2% cross-reactivity of human VEGF in the mouse VEGF ELISA. Serial dilutions of the tumor homogenates were tested to determine the optimal homogenate dilution for each analyte. Absorption was measured at 450 nm using a multiwell plate reader (Synergy HT, Biotek Instruments Inc., Winooski, VT). Curve fitting and sample concentration calculations were conducted with Gen5 software (Biotek). Results were normalized based on protein concentration or tissue wet weight.

2.7. Statistical analysis

Statistically significant differences between groups were determined using GraphPad Prism software. For all analyses, $p < 0.05$ is considered statistically significant. When comparing two groups, *F*-test for equality of variance was used to determine if the variance differed significantly. If variance between the two groups was equal, Student's *t*-test was used. For non-equal variance, non-parametric Mann–Whitney was used as indicated. To compare more than two groups, a significant main effect by one-way ANOVA was followed by post hoc Newman–Keuls analysis. Tumor volume over time was analyzed using a two-way repeated measures ANOVA, and significant main effects or interactions were analyzed using Bonferroni's post hoc analysis.

3. Results

3.1. Social isolation prior to MB-231 tumor cell injection

We have investigated the impact of stressor exposure in the form of social isolation on orthotopic growth of the human breast tumor cell line, MB-231, in SCID female mice. Mice were separated into single housing one week prior to MB-231 tumor cell injection (2×10^6 cells) into the mammary fat pad. Fig. 1 represents results from three experimental repetitions in which tumor growth was measured over time. In these experimental repetitions, tumor NE, human and mouse VEGF and IL-6 were not consistently altered at the time of sacrifice (day 83 post-MB-231 injection in Fig. 1; data not shown). No difference in lung metastases (the only site of metastasis from the primary MB-231 tumor) was observed between the two groups (data not shown). We postulated that the inability to produce replicable changes in tumor growth was due to the fact that MB-231 is a slow growing tumor *in vivo*, allowing mice to adapt to social isolation. If true, we predicted that social isolation would have a greater impact if transfer to single housing took place closer to the exponential phase of tumor growth.

3.2. Social isolation after MB-231 injection

To test this possibility, SCID mice were injected orthotopically with MB-231 (4×10^6 cells) and tumor growth was monitored over time. One-half of the mice were singly housed when tumors were palpable in all mice (in this experiment, day 14 post-tumor injection; average tumor volume = ~25 mm^3). The other mice remained in their home cages, and tumor growth in all mice was measured over time. MB-231 tumor growth was greater in singly-housed compared to group-housed mice when tumor volume was analyzed through day 28 post-separation (Fig. 2A; repeated measures ANOVA, main effect of housing, $p < 0.03$ with no interaction by time, $p = 0.5$). By day 34 post-separation, the effect of social isolation had dissipated somewhat. When this time point was included in the analysis, the main effect was no longer significant ($p = 0.07$) with no interaction by day post-separation ($p = 0.6$). Mice were sacrificed at this time point (day 34 post-separation). Tumor weight did not differ between groups (data not shown). Interestingly, a trend towards reduced tumor NE in the singly-housed group was noted (Fig. 2B, Mann–Whitney, $p = 0.1$). Tumor human VEGF concentration did not differ between groups (data not shown), but a trend towards increased human IL-6 was noted in tumors from singly-housed mice (Fig. 2C; Mann–Whitney, $p = 0.1$). Mouse IL-6 did not differ between groups (data not shown). No difference in lung metastases was observed between the two groups (data not shown). These results demonstrate that social isolation facilitated tumor progression, but the duration of the effect on tumor growth was limited.

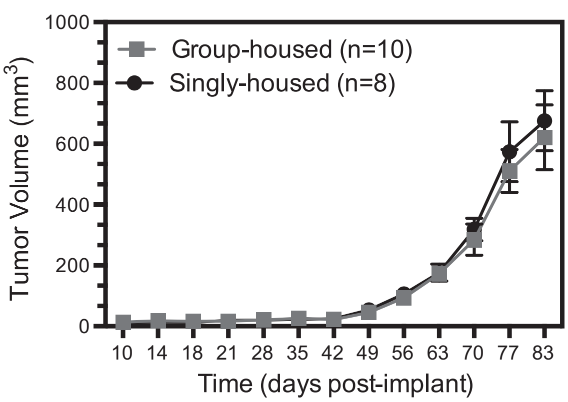

Fig. 1. MB-231 tumor growth is not altered by social isolation prior to tumor cell injection. SCID female mice were singly housed seven days prior to orthotopic injection of MB-231 cells. Tumor diameter was measured with calipers on the days indicated. Tumor volume is expressed as mean ± SEM, n = 8–10 mice per group.

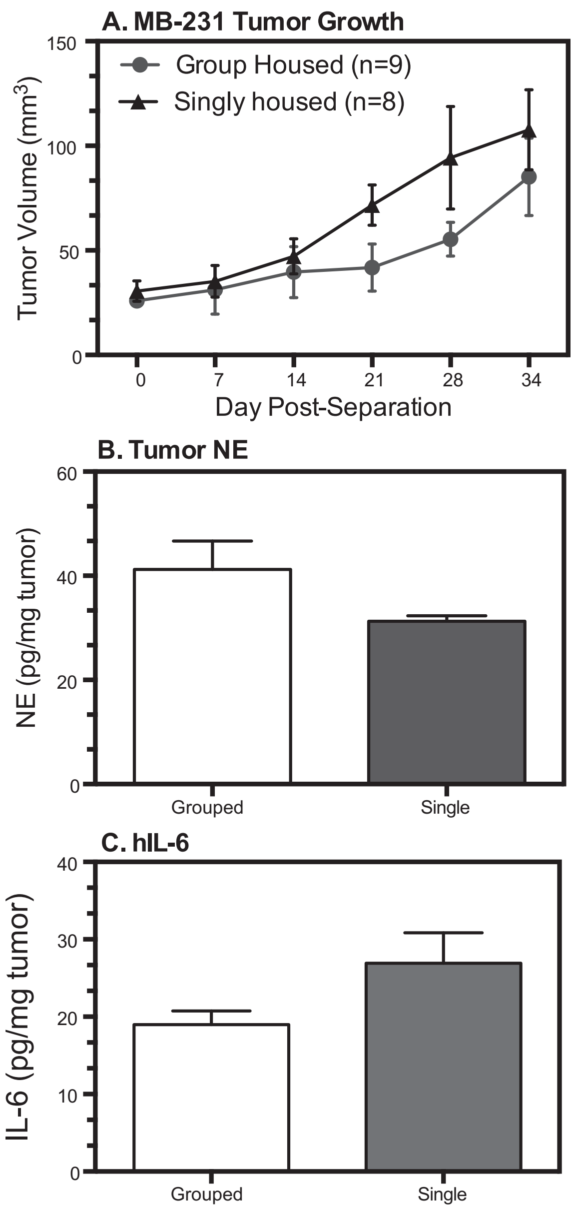

Fig. 2. MB-231 tumor growth is transiently increased by social isolation after tumor injection. SCID female were injected with MB-231 cells, and when all mice had palpable tumors, half of the mice were transferred from group to single housing. (A) Tumor volume over time. NE (B) and human IL-6 (C) were measured by ELISA in tumors harvested at day 34 post separation. Results are expressed as mean ± SEM, n = 8–9 mice per group. See text for statistical analysis of tumor growth. For tumor NE and human IL-6, no significant effects based on the non-parametric Mann–Whitney test, $p = 0.1$.

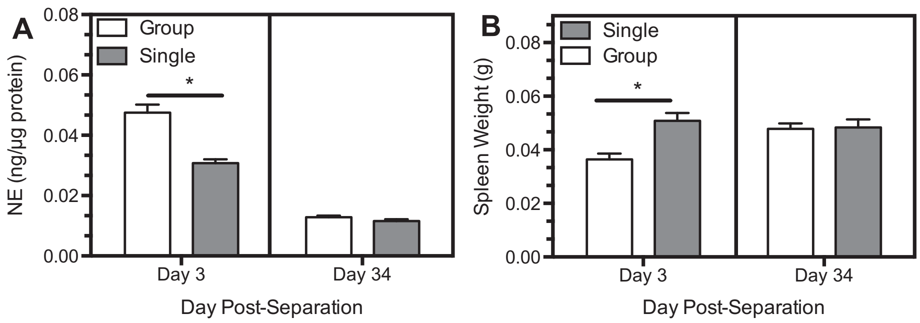

Fig. 3. Social isolation reciprocally alters spleen NE (A) and spleen weight (B) early after social isolation. A subset of mice described in Fig. 2 was sacrificed at day 3 post-separation. Results are expressed as mean ± SEM, $n = 5$ mice per group. Asterisk indicates different versus group-housed at the corresponding time point by Newman–Keuls post hoc analysis.

In this same experiment, a subset of mice was sacrificed day 3 after social isolation to determine if sympathetic activation occurred early after social isolation. At this time point, the tumors (⩽10 mg in weight) were too small to measure NE; therefore the spleen was used as a highly-innervated surrogate organ to compare NE concentration after social isolation. Day 3 post-separation, splenic NE concentration was significantly reduced in the singly-housed mice (Fig. 3A; ANOVA, housing x day interaction, $p < 0.001$). The decrease in NE concentration was associated with increased spleen weight (Fig. 3B; ANOVA, housing x day interaction, $p = 0.02$), suggesting that the increase in spleen mass reduced NE concentration in the singly housed mice. By day 34 post separation, neither NE concentration nor spleen weight was altered in singly-housed compared to group-housed mice, but compared to day 3, splenic NE concentration was significantly reduced in both groups in conjunction with increased spleen mass (ANOVA, main effect of day, $p < 0.001$).

3.3. Effects of social isolation on increased spleen mass are not dependent on tumor

In the next experiment, to determine if the early effect of social isolation on spleen mass was dependent on the presence of growing tumors, non-tumor bearing SCID female mice were socially isolated. In addition, flow cytometry was used to determine if macrophage populations were altered after social isolation, as reported for other social stressors (Engler et al., 2004). Social isolation increased spleen weight at this time point in association with decreased spleen NE concentration (Fig. 4A and B), similar to the effects at d3 in tumor-bearing mice. The percentage of splenic macrophages expressing F4/80+ and CD11b + Gr-1-cells was not significantly altered (Fig. 4C and E), but the percentage of CD11b+ macrophages that co-express Gr-1+ were significantly increased in socially isolated mice. These cells are myeloid derived suppressor cells that have potent immunosuppressive capabilities (Gabrilovich and Nagaraj, 2009). These results demonstrate that the early effect of social isolation in the spleen is independent of tumor growth, and that specific splenic macrophage populations are sensitive to neurohormonal changes elicited by social isolation. Since these macrophage populations are important regulators of tumor progression, we tested if macrophage populations are altered in spleens and tumors of socially isolated mice.

3.4. Social isolation alters spleen and tumor macrophage populations

The next experiment examined alterations in spleen and tumor NE concentration and macrophage populations 10 days after social isolation in mice bearing tumors. Social isolation was initiated when average tumor volume was 50 mm^3. Social isolation did not alter spleen or tumor weight or NE concentration 10 days after

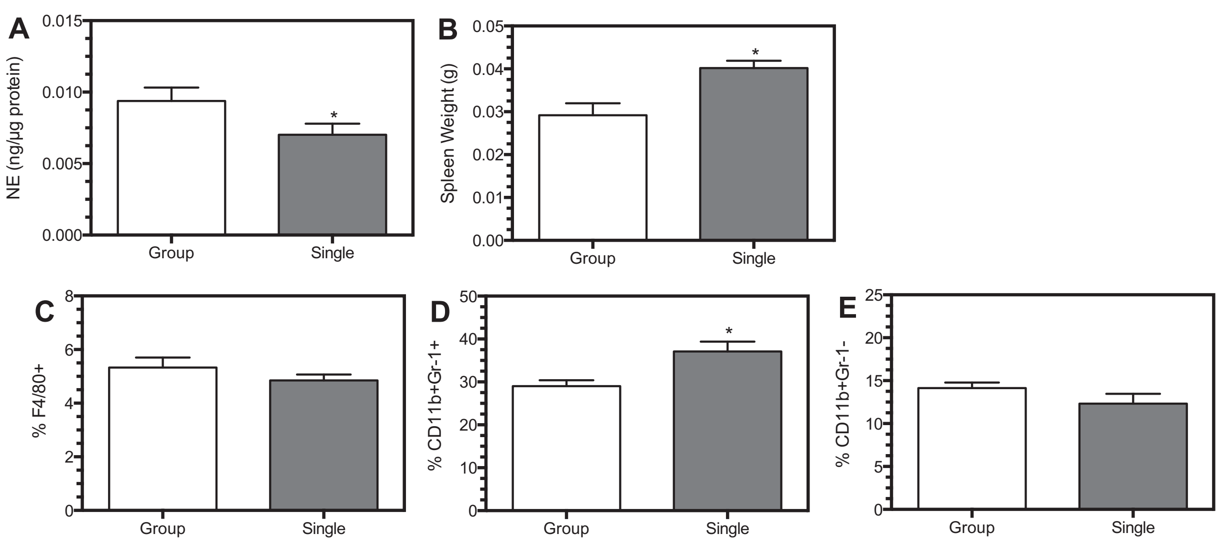

Fig. 4. Early effects of social isolation on spleen NE and spleen weight is independent of tumor growth. Three days after transfer to single housing, SCID female mice were sacrificed and spleen NE (A), weight (B), and macrophage populations (C–E) were determined. Asterisk indicates significant difference by Student's t-test, $p < 0.05$. Results are expressed as mean ± SEM, $n = 5$ mice per group.

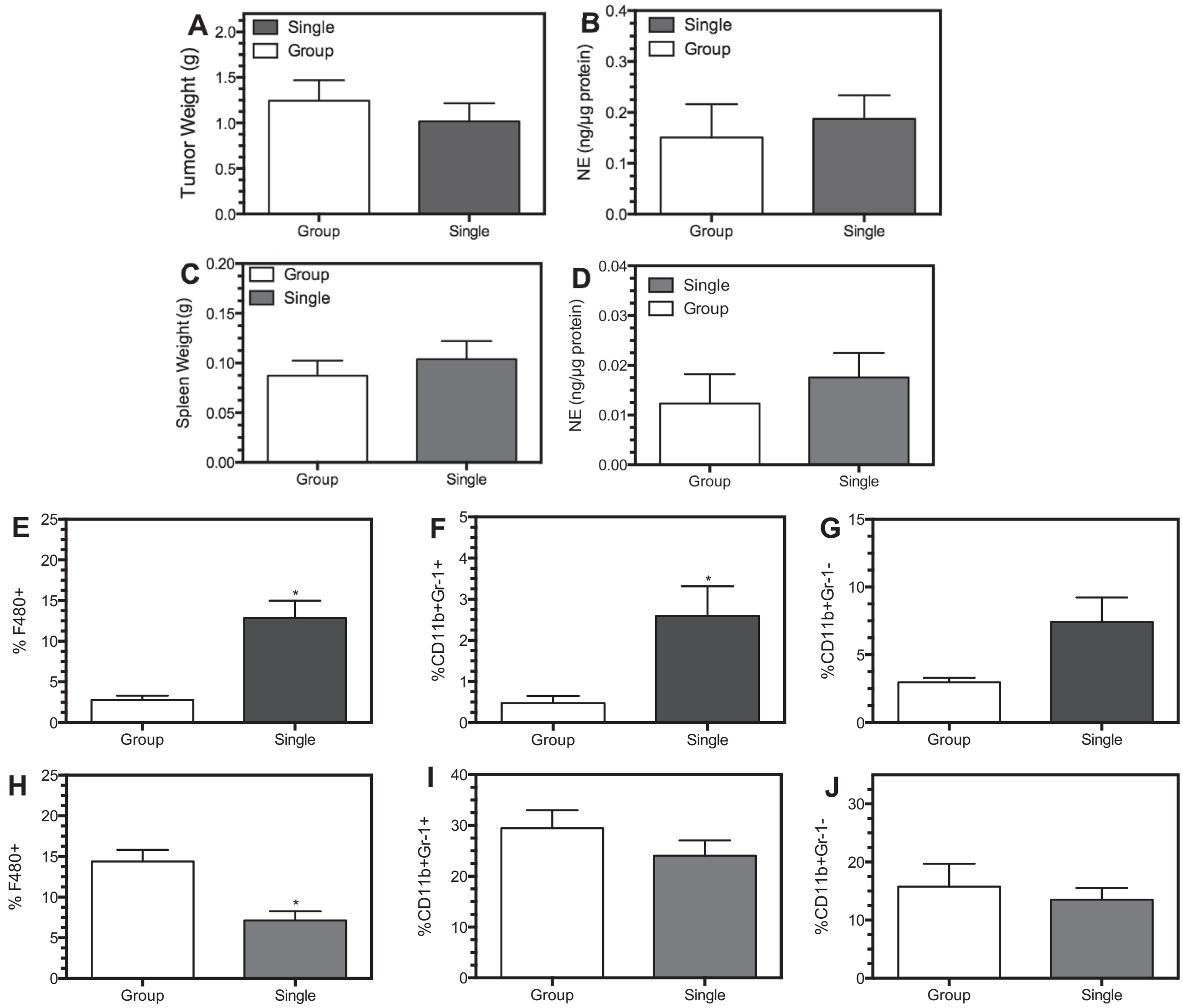

Fig. 5. Social isolation alters tumor and spleen macrophage populations. SCID female mice were injected with MB-231 in the mammary fat pad. When tumors were palpable, one-half the mice were transferred from group-housing to single housing. After 10 days, mice were sacrificed and tumor (A and B) and spleen (C and D) weight and NE concentration were determined, and macrophage populations were analyzed by flow cytometry in tumors (E–G) and spleens (H–J). Asterisk indicates significance based on the non-parametric Mann–Whitney test (A and B) or by student's *t*-test (D), $p < 0.05$. In (C), $p = 0.067$. The results are expressed as mean ± SEM of 9–10 mice per group.

social isolation (Fig. 5A–D). However, macrophage populations were increased in tumors from socially isolated mice with significant increases in the percentage of F4/80+ (Fig. 5E) and CD11b + Gr-1+ populations (Fig. 5F) and a trend toward an increase in the CD11b + Gr-1- population (Fig. 5G). In the spleen, the percentage of F480+ macrophages was reduced (Fig. 5H) with no significant changes in either CD11b+ population (Fig. 5I and J). These results demonstrate distinct changes in tumor and spleen macrophage populations with social isolation.

4. Discussion

Social isolation is a well-characterized social stressor that has several advantages to more standard laboratory-type stressors. It is a milder form of stress compared to other forms of stressor exposure. For example, in our hands singly-housed mice do not lose weight (data not shown) nor display elevated tissue NE concentration, in contrast to the weight loss (Sloan et al., 2010) and increased tissue NE concentration (Thaker et al., 2006) reported with daily restraint stress. Yet animals exposed to social isolation mimic behavioral anxiety and increased vigilance associated with social isolation observed in humans (Hermes et al., 2009; Williams et al., 2009). Here, we demonstrated that social isolation transiently increased MB-231 tumor growth, but only when social isolation was initiated when tumor growth was near exponential phase. No consistent effects of tumor growth or metastasis were observed when social isolation occurred prior to MB-231 injection, suggesting a temporal dependence in the context of a mild stressor. Indeed, early and transient changes in spleen NE concentration and macrophage populations, independent of whether or not the animals were tumor-bearing, were observed. Furthermore, increases in tumor macrophage populations took place before any indications of altered tumor growth. The changes in macrophage populations in the tumors were not associated with altered tumor NE. These results suggest that social isolation can have an impact on tumor progression, but the impact is transient and may not be associated with dramatic changes in tumor growth and metastasis.

The changes in macrophage populations in tumor and spleen indicate that social isolation facilitates leukocyte recruitment – a process described with another social stressor, social disruption

(Engler et al., 2004). Repeated social disruption increased spleen weight in concert with loss of CD11b+ myeloid cells from the bone marrow and an increase in CD11b+ cells in the spleen. Furthermore, the increase in spleen weight elicited by social disruption was mediated through β-AR stimulation, as it was prevented by propranolol pretreatment to block β-AR (Wohleb et al., 2011). The increase in the CD11b + Gr-1+ population in spleen from non-tumor bearing, singly-housed mice shown here (Fig. 4), suggests that this process also occurs with social isolation. A similar stress-induced increase in tumor macrophages has been described by Sloan and colleagues, who demonstrated elevated F4/80+ tumor macrophages and a trend toward increased myeloid derived suppressor cells with restraint stress; this effect was blocked by propranolol treatment (Sloan et al., 2010). Restraint stress did not facilitate primary tumor growth, but increased tumor angiogenesis and dramatically elevated metastasis (Sloan et al., 2010). In our hands, tumor associated macrophage populations expressing F4/80 and CD11b were increased with social isolation. The spleen is an important source of tumor associated macrophages and tumor associated neutrophils (Cortez-Retamozo et al., 2012). The social isolation-induced decrease in the splenic F4/80+ population in conjunction with an increase in this population in tumors suggests that the spleen may contribute to the increased F4/80+ tumor associated macrophages in socially isolated mice. It is likely that both the spleen and bone marrow may be targets of stress hormones that promote the migration of these macrophage populations into the tumor. Both tumor associated macrophages and myeloid derived suppressor cell populations are associated with tumor progression (Gabrilovich and Nagaraj, 2009), but we have yet to establish that the increased tumor macrophages lead to the increased tumor growth in the singly-housed mice.

Social isolation has been characterized as a stressor based on behavior (it elicits anxiety behaviors in female mice) (Palanza et al., 2001), but is less well characterized in terms of hypothalamic pituitary axis or sympathetic nervous system activation. Long-term social isolation increased development of spontaneous mammary tumors and metastasis, but these rats were socially isolated from puberty (Hermes et al., 2009), making it difficult to directly compare to the social isolation procedure here, which was begun when the mice had reached adulthood. Nonetheless, social isolation led to reduced baseline levels of plasma corticosterone at the nadir of the diurnal rhythm in social isolated rats, but an elevated and prolonged corticosterone response to a stressor (Hermes et al., 2009; Williams et al., 2009). Similarly, a 21-day period of single housing did not alter baseline plasma catecholamines, but upon exposure to an acute stressor, plasma catecholamines in socially isolated rats were significantly elevated versus group-housed (Dronjak et al., 2004). Therefore, the social isolation model will be particularly useful for examining the impact of an acute stressor in animals exposed to long-term social isolation. The results presented here suggest that social isolation alone may elicit alterations that have a transient impact. Future plans include using social isolation to understand the biological consequences of multiple stressors on tumor pathogenesis, a more likely scenario in the context of a diagnosis of breast cancer.

The effects of social isolation were not associated with increased NE concentrations in spleen or in tumor, as might be expected if social isolation activated the SNS. However, there are a few caveats in interpreting tissue NE measures. First, NE concentration in the spleen appeared to fluctuate with changes in spleen mass. One way to interpret this finding is that sympathetic nerve fibers within the spleen do not respond rapidly to a rapid expansion in tissue volume, such as the increase in spleen weight with social isolation or even in a growing tumor. We can detect sympathetic nerve fibers in MB-231 tumors independent of tumor size, however the impact of the expanding tumor architecture on NE concentration has not been systematically examined. Furthermore, measuring only tissue NE may not be an appropriate measure of SNS activation, especially under conditions of a relatively mild stressor such as social isolation where homeostatic mechanisms serve to maintain a constant tissue NE baseline (Eisenhofer et al., 2004)). Therefore, we have begun to assess normetanephrine, a product of NE metabolism by catechol-*O*-methyltransferase, as a potential additional measure of sympathetic activation and released NE. These experiments will help define the role of SNS activation and β-AR stimulation in the context of social isolation.

The results presented here demonstrate an early, but transient effect of a psychological stressor, social isolation, in both tumor-bearing and normal female SCID mice. The results imply that exposures to relatively mild stressors may promote tumor progression, depending on the timing relative to tumor growth, but also suggest the possibility that homeostatic mechanisms can mitigate the impact of social isolation. This is a potential area of investigation in terms of identifying pathways that help minimize the impact of chronic stress experienced in breast cancer patients. It is critical to understand how mild stressors interact to develop into a more severe stressor and to develop therapies that work in concert with standard breast cancer therapies to inhibit tumor progression.

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Conflict of Interest

The authors of this manuscript have nothing to declare.

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